The Effect of Increasing Carbohydrate Concentrations on Expressed Intracellular and Extracellular Proteins by the *Photorhabdus luminescens* Bacterium and the Relative Bioluminescent Output

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Abstract

Photorhabdus luminescens is a Gram-negative, pigment-producing enteric bacterium, bioluminescent and pathogenic to insects, with the capability to undergo phase variation. *P. luminescens* is symbiotically associated with the soil-dwelling nematode, *Heterorhabditis bacteriophora*, while also playing a crucial role in the reproduction cycle of this nematode. The phase I variant of *P. luminescens* produces light energy in the form of bioluminescence, the red anthraquinone-derived pigment, a symbiotic trait infective juveniles of *H. bacteriophora* exploit to attract insect hosts. The current study examined the effects of different carbohydrates at various concentrations on the growth of the *P. luminescens* phase I variant and the production of bacterial luminosity in liquid culture, while also examining the relative amounts of expressed extracellular and intracellular proteins. In this study, 2x Nutrient broth was used as a liquid growth media. Bioluminescence was measured with a Modulus™ single tube luminometer (Turner Biosystems) and reported in terms of relative luminosity units (RLU). Protein assays were performed using a spectrophotometer (Beckman™ DU640) set at a wavelength of 595nm, while utilizing Bradford dye. Bacterial growth density was also measured at 600 nm using a spectrophotometer (Beckman™ DU640). The carbohydrates utilized in the study of expressed extracellular and intracellular proteins were trehalose, cellobiose and d-sucrose, at 1, 3, 5 and 7 %. Overall bioluminescence and absorbance data utilized samples grown in varying concentrations of D-sucrose, Cellobiose, and Trehalose. The concentrations were varied from 1 % to 7 %.

Keywords: Microbiology, Entomopathogenic Nematodes, Bacterial density, Soil-dwelling bacteria

Introduction

Bioluminescence is directly related to the bacteria's ability to infect an insect host (Patterson et al., 2015). Previous research has suggested a correlation between relative distance between bioluminescent bacterial cultures and insects (Walter et al., 2015). As bioluminescent output increases, distance between pathogenic bacteria and insect hosts decrease allowing for a better understanding of the role of luminescence within the bacterial colonies, and specifically how it is an indication of the critical and necessary symbiotic relationship with its counterpart, *Heterorhabditis bacteriophora*, a known entomopathogenic nematode (EPN).

The bacteria samples must complete their life-cycle in the gut of a soil dwelling nematode *H. bacteriophora* (Patterson et al. 2015). The nematode referred to is known as *Heterorhabditis bacteriophora, a* cruiser-type species of nematode, which utilize their movement capabilities to seek out their hosts. Once a host is infected, the nematodes will lay eggs, which will hatch and then exit the insect carcass to seek out new hosts to infect. *H. bacteriophora* is known to inhabit the deeper regions of the soil where pH is highly variable an does not remain constant (Stuart et al., 2015). However, it is known that nematodes can generally tolerate soil types varying in pH while also being affected by relative levels of O_2 and N (Kung et al., 1990).

The relationship between *H. bacteriophora* and *Photorhabdus luminescens* is mutualistic; (Patterson et al. 2015). Once the soil-dwelling nematode finds an insect host, the nematode penetrates the insect to release the bacteria into the hemocoel (Forst and Nealson, 1996). To be more specific, the ability of *P. luminescens* to infect other detrimental insects is dependent upon the completion of a full life cycle within the nematode, *H. bacteriophora.* The nematode provides this bacteria with mobility (Stuart et al., 2015); something that it would otherwise not have without the symbiotic relationship with the nematode, *H. bacteriophora.* This bacteria will

develop the ability to secrete digestive enzymes and insecticidal toxins that work to kill the insect as the bacterium continues to be virulent and remain in symbiosis the nematode (Daborn et al., 2002).

P. luminescens is carried within the intestinal tract of Infective Juvenile (IJ) *H. bacteriophora.* Infective juveniles can penetrate insects through natural openings and the tegument, causing death of insects in the larval and adult stages. Bacteria are released into the hemocoel of the insect where they can divide. For proper nematode growth and development within the hemocoel of the insect, *P. luminescens* bacteria are required. Infective juveniles carrying *P. luminescens* leave the insect carcass and seek out a new host to complete their life cycle (Forst et al., 1997).

This bi-phasic process requires the use of many enzymatic processes and abilities to secrete toxins to kill the insect and complete symbiosis (Daborn et al 2002). Many of these toxins and enzymatic processes, some of which attribute to the bacterium's ability to kill the insect host within 24-48 hours, have been identified (Bowen et al. 1998). *P. luminescens* also has been shown to produce other compounds such as crystalline inclusion proteins and proteases (Dunphy and Webster, 1988), and also insecticidal toxins (Bowen et al. 1998), that illicit the degradation of the insect host

A novel genome sequence of *P. luminescens* encoding a large number of the pathogenic toxins produced has been published and recognized. The gene sequence encodes several proteins involved in the virulence factor of this bacteria. The gene sequence, known as TT01, is 5,688,987 base pairs long and encodes an estimated 4,839 specific coding genes for proteins (Duchaud et al., 2003). This gene sequence revealed that virulent proteins encoded by the genomic sequence of *P. luminescens* kill the insect through septicemia or toxemia following the

initial life cycle stage within the gut of the IJ. The life cycle is composed of two parts: a symbiotic stage when the bacteria grow in the insect gut, and the infective phase in which the bacteria effectively kills the insect (Duchaud et al. 2003).

This bacterium undergoes phase variation. Phase variation refers to a bacteria's relative ability to modulate its phenotypic expression, *in vivo,* due to external environmental factors (Henderson et al., 1999). The phase I variant is significant because it has the supports the life cycle of the *H. bacteriophora*. Phase II variations of the bacterium do not have the ability to support growth of this nematode *in vivo* or *in vitro* (Holmes et al. 2012). The phase I variation of this bacterium will be utilized for experimentation.

Previous research has shown that Trehalose, a naturally occurring disaccharide of two glucose molecules, is naturally abundant in insect hemolymph. Trehalose has shown a unique ability to increase the stability of this organism *in vivo* an *in vitro* , also showing that increased concentrations of Trehalose lead to higher bioluminescent outputs of *P. luminescens in vitro* (Holmes et al., 2012). Increased bioluminescent outputs are positively correlated with the bacteria's ability to attract an insect, *in vivo,* (Patterson et al., 2015). In lepidopterous insects, Trehalose has been shown to comprise 90 % of the blood sugar samples (Wyatt, 1957). Trehalose is also known to serve as a non-reducing agent that is also a source of carbon and energy; while it is also thought to be a type of signaling molecule for other metabolic pathways for growth (Elbein et al., 2003).

It was hypothesized that if increased carbohydrate concentrations would affect this bacterium's bioluminescent output, growth rate, and overall expression of protein(s); however, it was unknown what carbohydrate(s), or at what carbohydrate concentration, *P. luminescens* would interact positively or negatively. The use of EPN's as biocontrol agents are also growing

at an ever increasing rate in the US (Ehlers and Hokkanen, 1996), as well as parts of European biocontrol industry (Ehlers, 2003). The importance of further research into optimizing certain conditions for EPN's, as well as the *P. luminescens* bacterium, is crucial to understanding the possibilities this organism has for the Agriculture industry worldwide.

Methods

Bacterial Sample Preparation

To measure the effects of carbohydrates on *P. luminescens,* carbohydrate solutions must be prepared with known carbohydrate concentrations and aseptic techniques. Carbohydrate concentrations ranged from 0 %-7 % in each respective flask containing nutrient broth. Carbohydrates used were Trehalose, D-sucrose, Cellobiose, D-Sorbitol and Maltose. Carbohydrate's was dissolved in 5 mL of sterile-deionized water, using an agitator for 25-30 seconds, until the solution was free of large particles.

The liquid carbohydrate solution was injected into the respective beaker that contained the nutrient broth solution. Ten to twenty microliters of bacterial stock was transferred into each labeled flask according to carbohydrate and concentration. Flasks containing bacterial samples were placed in an incubator shaker at a temperature of 37 ^{o}C for 24-48 h, undergoing mild agitation at a low RPM.

Bioluminescence and Absorbance Data Measurement

Bioluminescence was measured using a single tube luminometer (model #, company name, city) to measure luminescence in relative luminosity units (RLU). Changes in bacterial density and relative protein concentration(s) were measured and observed using a spectrophotometer set at a wavelength of 600 nm.

Samples to be used for measuring luminescence were placed in 5 mL graduated microtubules (model #, company name, city) compatible with the luminometer.

Protein Sample Preparation

Intracellular protein

In order to isolate intracellular proteins, the bacterial cell wall must be broken down and lysed open to expose inside cellular material (Harrison, 1991). Five milliliters of bacteria were collected in a sterile 15 mL tube and spun down in a centrifuge at a speed of 10,000 RPM for 15 min, at a temperature of 4 ºC to ensure protein(s) within sample did not denature. Following initial centrifugation process, the supernatant was discarded and the pellet containing bacterial cells were re-suspended in 1 mL of bacterial cell lysis buffer. The sample was hegemonized for the lysis buffer to function efficiently and allow for lysis of all bacterial cell walls. Samples were centrifuged again for 15 min at 10,000 RPM at 4 ºC. Following the second centrifugation cycle, the supernatant was collected in new, sterile 15 mL conical tube and immediately placed in a -20 ºC freezer. Samples were thawed and used at a later time for protein assay and data collection.

Extracellular protein

To isolate extracellular proteins, 5-10 mL was used centrifuged to attain a supernatant and pellet. The pellet in the isolation of extracellular proteins was waste and was not be used in the collection process. The supernatant was collected because it should have contained only extracellular proteins that were excreted by the bacteria. Supernatant was transferred to new vial upon completion of isolation process. Samples were either immediately used for experimentation following preparation, or stored at -20ºC

Extracellular and Intracellular Protein Assay and Data Collection

Bradford dye was used in conjunction with a spectrophotometer at a wavelength of 595 nm to measure and record relative protein concentrations within a sample. Though the protocol for extracellular and intracellular protein isolation differs in many ways, the protocol for measuring protein concentration was the same. A standard curve was established that was used to calculate protein concentration (µg/mL). An R^2 value must be >0.95 to ensure more accurate values.

Results

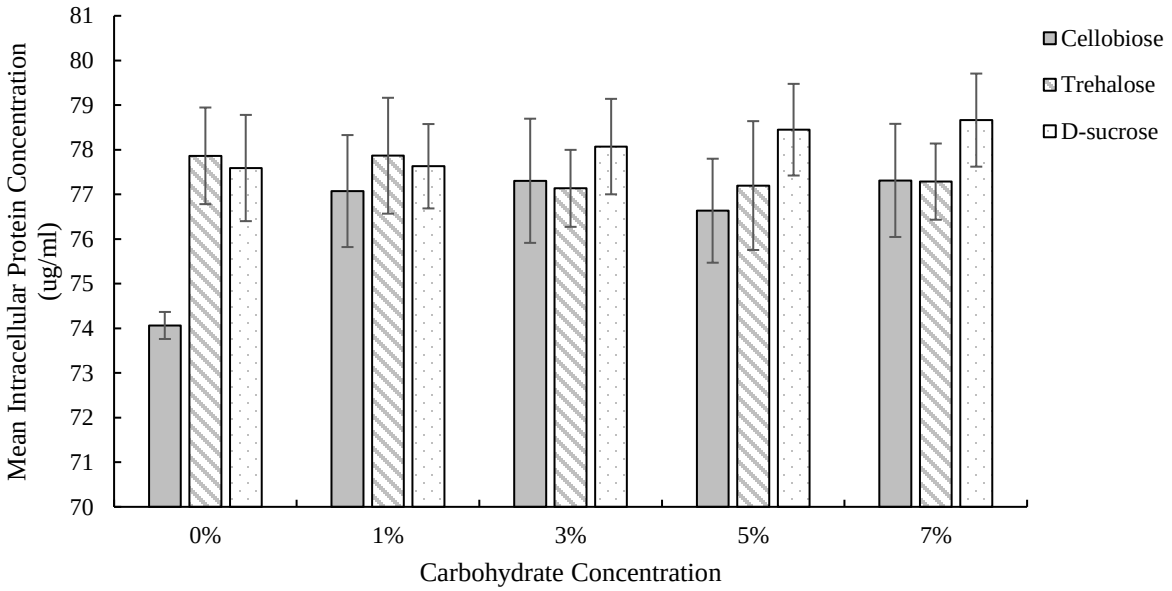

Figure 1. The effects of increasing carbohydrate concentration on expressed intracellular proteins from *Photorhabdus luminescens.* Each treatment group was inoculated with the same number of bacteria before growth phase. Results show the expressed amount of intracellular proteins within each sample following Bradford Assay. Significant differences were observed when comparing Cellobiose and D-Sucrose treatments (p=0.02), and when comparing Trehalose and D-Sucrose treatments (p=0.03). Two sample T-tests were used to determine any statistical significance.

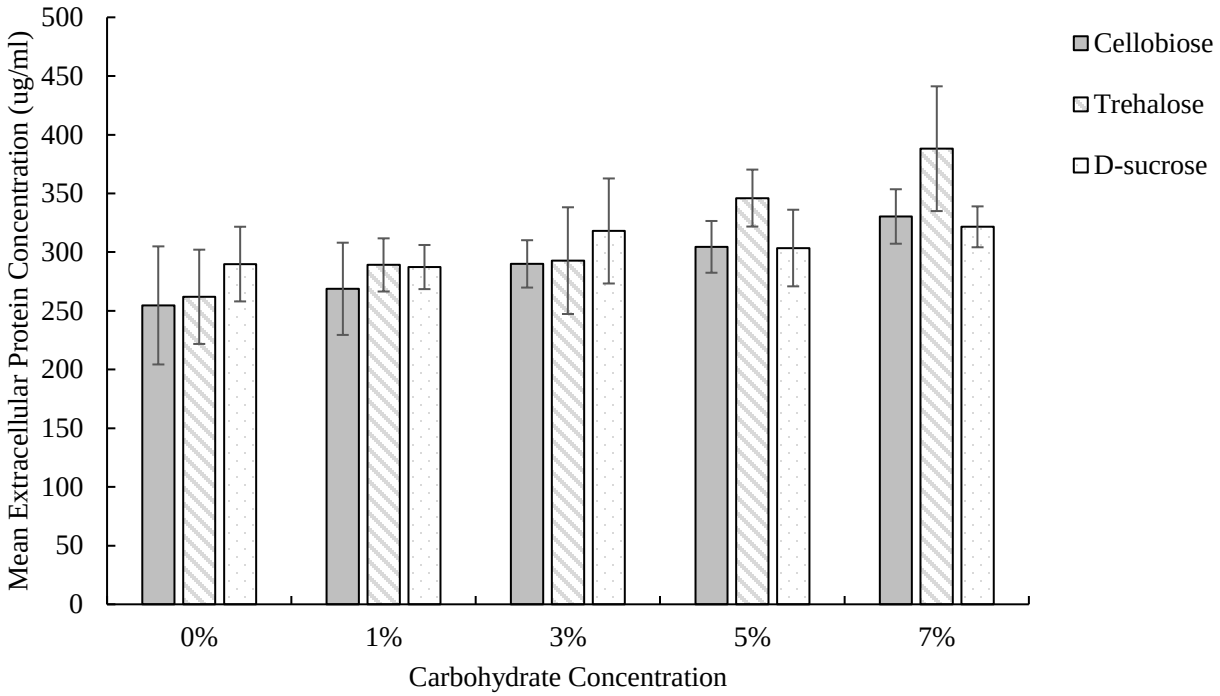

Figure 2. The effects of increasing carbohydrate concentration on expressed extracellular proteins from *Photorhabdus luminescens.* Each treatment group was inoculated with the same amount of bacterial sample before growth phase. Treatment groups involved that of varying concentrations of Cellobiose, Trehalose and D-Sucrose. Concentrations varied from 0% to 7%. Results show the expressed amount of extracellular proteins within each sample. Significant differences were not observed when comparing Cellobiose and D-Sucrose treatments ($p=0.18$), Trehalose and D-Sucrose treatments ($p=0.32$), or Cellobiose and Trehalose ($p=0.18$).

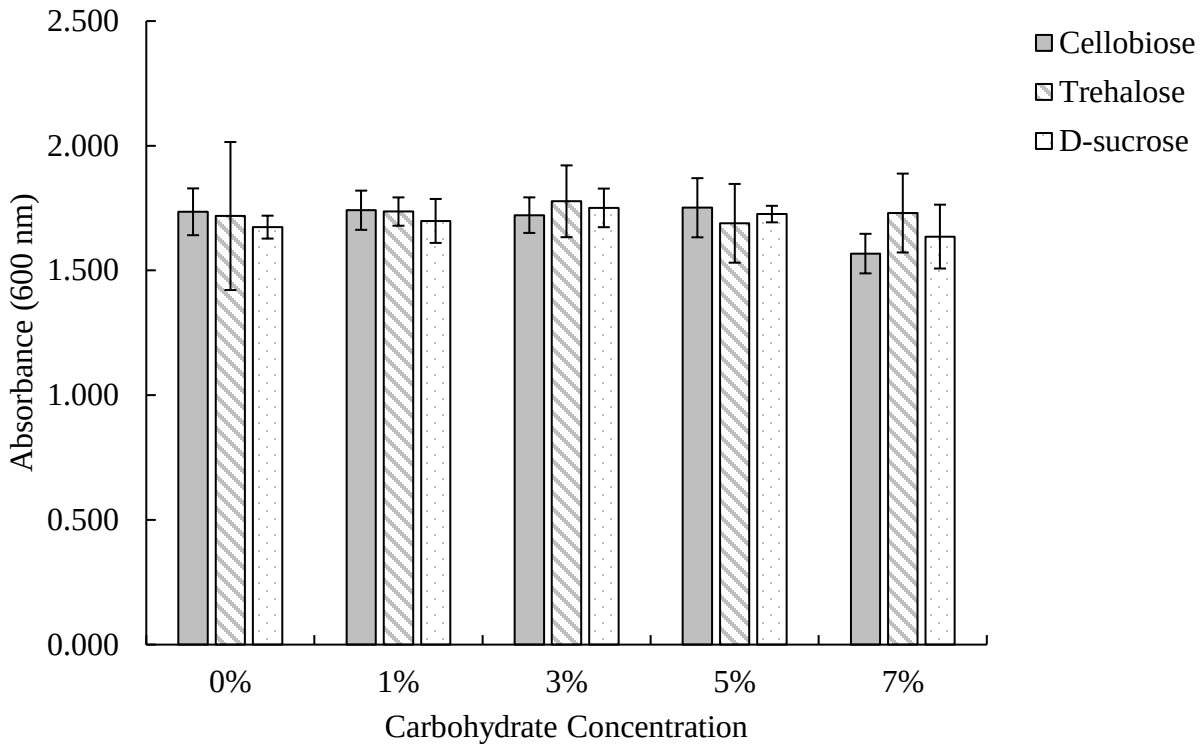

Figure 3. The effects of increasing carbohydrate concentration on the bacterial density of samples from *Photorhabdus luminescens.* Each treatment group was inoculated with the same amount of bacterial sample before growth phase. Absorbance measurements were taken at a wavelength of 600 nm following a growth period of 48 hours. Results show the relative absorbance readings of each respective sample, with no significant differences observed between groups ($p>0.11$). Error bars show ± 1 s.d.

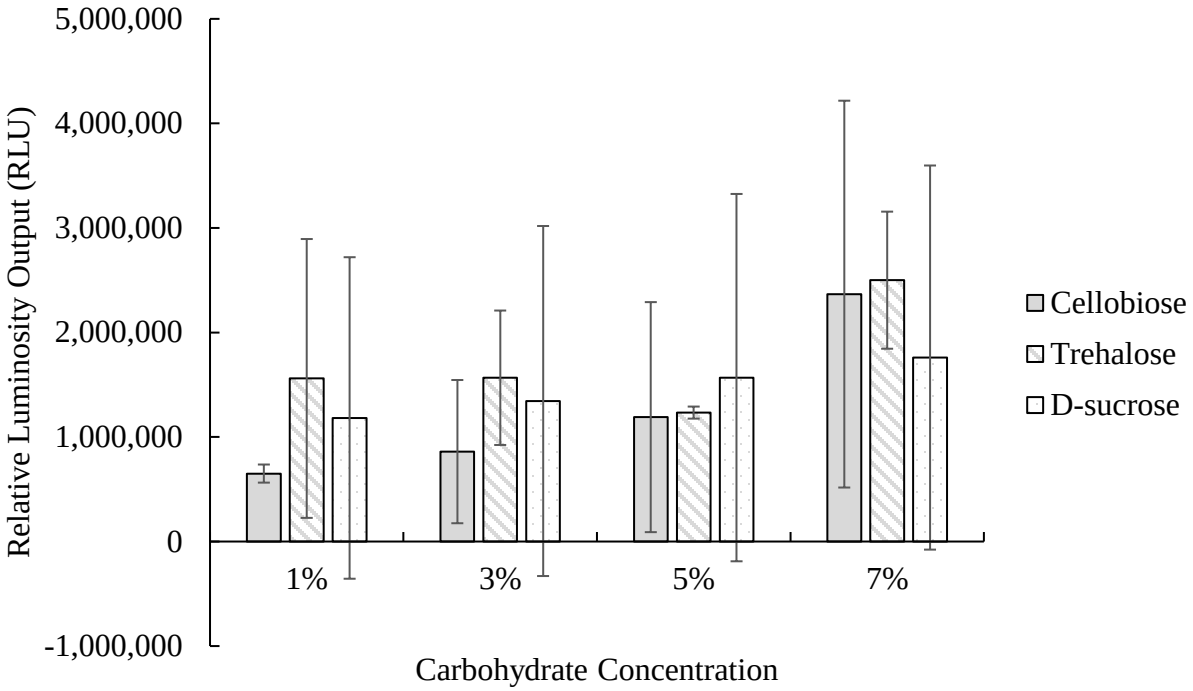

Figure 4. The effects of increasing carbohydrate concentration on the bioluminescent output of *Photorhabdus luminescens* bacterial samples. Each treatment group was inoculated with the same amount of bacterial sample before growth phase. Bioluminescent output was measured following a growth period of 48 hours. No significant differences were observed between groups ($p>0.19$). Error bars show ± 1 s.d.

Results

Intracellular and Extracellular Proteins

Data collected from measuring the expressed amount of intracellular proteins did not support the hypothesis that increasing carbohydrate concentrations would positively affect the expression of intracellular proteins. Though, there was an obvious increasing trend from 0 % concentrations for Cellobiose and Trehalose treatments, there was not a uniform trend as the concentration increased to 7 % (Fig. 1). Cellobiose showed an increase in intracellular protein concentration from 74 µg/ml at 0 % carbohydrate concentration to 77 µg/ml at 7% cellobiose concentration. Significant differences were discovered when comparing Cellobiose and D-Sucrose treatment groups ($p=0.02$), as well as when comparing Trehalose and D-Sucrose treatment groups ($p=0.03$) when using a two sample T-test.

Extracellular protein concentration data did not show any significant trends to support that the hypothesis that increasing carbohydrate concentrations would increase the overall expression of extracellular proteins. However, there was an increasing trend in the extracellular protein centration from 0 % Trehalose concentrations,261 ± 40 µg/ml, that increased to 388 ± 53 µg/ml at a concentration of 7 % Trehalose (Fig. 2). Trehalose treated groups showed the greatest increase from 0 %, however, Cellobiose and D-Sucrose treated groups also showed an increasing trend from 0 % concentrations. There were no significant differences observed when comparing the overall expression of extracellular protein in cellobiose, Trehalose, and D-sucrose treated samples of *P. luminescens* ($p>0.05$).

Bacterial Density

Following an analysis of bacterial density of samples as carbohydrate concentrations increase, the hypothesis that growth rate would increase as a result of increasing carbohydrate concentrations was not supported. Cellobiose and D-sucrose samples decreased from 0 % carbohydrate concentration to 7 %. An absorbance value of 1.74 at 0 % was recovered for Cellobiose samples, that then decreased to a value of 1.57 at a concentration of 7 %, while D-sucrose showed an absorbance value of 1.68 at a 0 % concentration that decreased to a value of 1.64 at a 7 % concentration (Fig. 3). There were no significant differences observed among Cellobiose, Trehalose or D-Sucrose treated samples when comparing their bacterial density as carbohydrate concentrations increase ($p>0.05$).

Bioluminescent Output

The hypothesis that increasing carbohydrate concentration would increase the bioluminescent output of this bacterium was not supported. There is an increasing trend observed in Trehalose and Cellobiose treated groups, however, the variance in the data has standard error greater than most of the averages for each concentration. At 0 % Trehalose concentration, a bioluminescent output of 1,560,000 ± 1,333,716 was reported, which increased to an output of 2,500,000 ± 655,743 at a 7 % concentration (Fig. 4). The significant amount of variance in the data makes it difficult to establish any trends or sources of significance. There were no significant differences observed when comparing the elative luminosity output among Cellobiose, Trehalose, and D-Sucrose treated samples containing *P. luminescens* ($p>0.05$).

Discussion

The purpose of this study was to understand the effects of carbohydrates on bioluminescent output, bacterial growth, and relative expression of extracellular and intracellular proteins. *P. luminescens* is a luminescent bacterium that plays a large role in seeking out and killing insects as a biological pest control agent (Patterson et al., 2015). Data collected did not support the hypothesis that increasing carbohydrate concentrations would positively affect bioluminescent output, bacterial density, or expressed intracellular and extracellular proteins.

Intracellular protein concentrations did not increase with increasing carbohydrate concentrations, as originally hypothesized. Trehalose treated groups actually showed a decrease in protein concentration as carbohydrate concentrations increased (Fig. 1). Cellobiose treated groups had an increase from 74 µg/ml ± 0.3 at 0 % carbohydrate concentrations that increased to 77 µg/ml ± 1.3 at a carbohydrate concentration of 7%. Cellobiose treated groups displayed the greatest increase from a 0 % carbohydrate concentration, however, this increase does not seem to be affected by the concentration, but rather the presence of the carbohydrate (Fig. 1). There were statistical differences observed when comparing Cellobiose and D-Sucrose treated groups ($p=0.02$) and Trehalose and D-sucrose treated groups ($p=0.03$).

The hypothesis that increasing carbohydrate concentrations would positively affect expressed extracellular protein concentrations from the *p luminescens* bacterium was not supported. There was an increase from 0 % Trehalose at a protein concentration of 261 ± 40 µg/ml that then increased to 388 ± 53 µg/ml at a concentration of 7 % (Fig. 2). However, despite obvious increasing trends there was not any statistically significant differences when comparing

Trehalose and Cellobiose (p=0.17), Trehalose and D-Sucrose (p=0.32), or Cellobiose and D-Sucrose (p=0.18).

Bacterial density, measured using absorbance, was not affected by increasing carbohydrate concentrations among the three treatment groups. Absorbance values recorded remained between 1.5 and 2 among all carbohydrate treated groups. Absorbance values remained constant as the carbohydrate concentration values increased, except in the case of Cellobiose at a concentration of 7 %. At a Cellobiose carbohydrate concentration of 5 %, the absorbance value remained at 1.75 ± 0.12 that then decreased to 1.57 ± 0.08 at a carbohydrate concentration of 7 % (Fig. 3). Other carbohydrates used in this study did not show the same trends when measuring bacterial density at a wavelength of 600 nm. No statistical differences were observed when comparing treatment groups.

Previous studies have shown increased concentrations of Trehalose lead to increased luminescent outputs (Holmes et al. 2012). This was explained by the fact that there already exist certain enzymatic processes within this bacterium to more effectively break down Trehalose compounds compared to other carbohydrates that may be found in their environments. It is also important to note that Trehalose is one of the most abundant carbohydrate compounds found in nature.

Bioluminescent output did not increase with increasing carbohydrate concentrations, as was expected based on previous work with the *P. luminescens* and Trehalose showing that increasing Trehalose carbohydrate concentration has a positive effect on the relative bioluminescent output of the bacterium (Holmes et al., 2012). Data collected in this study conflict with these previous findings—trehalose treated groups did portray an increasing bioluminescent output as carbohydrate concentrations increased, however, the variance in the

data was problematic when determining the validity of the hypothesis. At a concentration of 0 % Trehalose, the bioluminescent output was recorded as 1,560,000 ± 1,333,716 RLU; that increased to 2,500,000 ± 655,743 RLU. At a 7 % Trehalose concentration (Fig. 4). The other carbohydrate treated groups also showed an increase as carbohydrate concentrations increased, however, the variance once again became an issue when attempting to support validity of the proposed hypothesis. No significant differences were observed when analyzing the data, leading to the conclusion that the data do not suggest a positive correlation with increasing carbohydrate concentrations and an increase in bioluminescent output.

It was also previously shown that 1.0 % Trehalose concentrations in liquid media maintain bioluminosity and pigmentation of *P. luminescens* a longer period of time compared to glucose-treated cultures (Floyd et al., 2012). The results of the current study did provide evidence that Trehalose can have a beneficial effect on bioluminescence in small concentrations. This study utilized concentrations that ranged above 1 %, possibly contributing to some of the major differences in observed results. The increased concentrations of these carbohydrates could have negatively impacted this bacterium's ability to effectively stabilize bioluminescent output. This could have been due to the excess concentrations of carbohydrate significantly changing the overall growth environment for the bacteria; negatively affecting the bacteria's ability to stabilize relative luminosity output. Previous work on the effects of carbohydrates on expressed intracellular and extracellular proteins is not readily available, however, work done to better understand the genes encoding the various proteins and toxic compounds utilized by this bacterium has been performed (Bowen et al., 1998).

The results of this study did not show that increased carbohydrate concentrations of Trehalose, D-sucrose, and Cellobiose had an effect on bioluminescent output, bacterial density,

or overall expressed extracellular proteins. The results did show, however, that increased carbohydrate concentrations have an effect on the overall expressed intracellular proteins, contrasting with previously published data. It is possible that there were some sources of error in this study that contributed to this inconsistency. At 0 % concentrations, when measuring expressed intracellular proteins, there should have been no observable difference in the amount of expressed intracellular proteins between control and treatment groups. However, as Fig. 1 shows, Cellobiose-treated groups have a significantly lower amount of expressed intracellular proteins when compared to D-Sucrose treated samples ($p=0.02$).

The bioluminescent output of each carbohydrate-treated group contain significant variance. Changes to the protocol for observing relative luminosity output is recommended to allow for more consistent data in future projects. The variance in the data could be attributed to differences in growth time, growth conditions, or other confounding variables that could affect the bacterium's ability to grow and replicate during the growth phase. More studies to determine various growth conditions on the bioluminescent output of *P. luminescens* are needed.

Further research should also investigate carbohydrates with similar compounds to Trehalose. Trehalose is a disaccharide molecule with an alpha, alpha-1,1 linkage bonding them together (Higashiyama, 2002). Compounds with similar structures could be used to understand if they function in a similar way due to similarities in their overall structure. Overall, more studies are required to understand more about the direct effects of carbohydrates on *P. luminescens*; specifically, as it relates to the mechanisms used to effectively utilize carbohydrates for energy

Conclusion

The purpose of this study was to understand the effects of increasing Trehalose, cellobiose and d-sucrose carbohydrate concentrations on the bioluminescent output, bacterial density and overall protein expression in the *Photorhabdus luminescens* bacteria. Previous studies have shown that increasing concentrations of Trehalose can positively influence *P. lumincencens* bioluminescent output *in vitro* (Floyd et al., 2012). Data observing the changes in intracellular and extracellular protein concentrations, with respect to increasing carbohydrate concentrations, did not yield strong evidence to support the claim that increasing concentrations of trehalose, d-sucrose, and cellobiose positively affects overall protein expression. However, there are many studies closely examining the effects of varying external conditions on *P. luminescens* symbiotic counterpart, *H. bacteriophora.* More specifically, it has been shown that EPN's respond to environmental changes in oxygen, pH, and other stimuli (Stuart et al., 2015). More data is needed to determine the relative changes in the response of *P. luminescens* under similar environmental conditions.

In this experiment, *in vitro* conditions were manipulated. By manipulating these conditions, we were able to re-create different environments of increasing concentrations of carbohydrates. It was hypothesized that *P. luminescens* would exhibit increased bacterial density, bioluminescent output, and increased overall protein expression. Data suggested that increased carbohydrate concentrations do not positively influence this bacteria's bioluminescent output or bacterial density (Fig. 3 & 4). Data also suggested that increasing carbohydrate concentrations have a positive influence on the overall expression of extracellular and intracellular proteins (Fig. 1 & 2). *P. luminescens* does respond to changes in carbohydrates concentration, as well as the type of carbohydrate used. However, it has not been determined if this response could be

precisely predicted when using a similar methodology and protocol used in this study. Overall, the results of this study express the notion that continued studies are needed to better understand the underlying mechanisms used by *P. luminescens* to effectively utilize carbohydrates *in vitro.*

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